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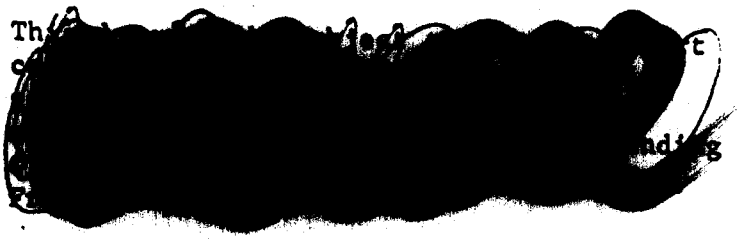

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THE NATURE OF THE TOXIN OF BACILLUS BOTULINUS

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The botulinus toxin is an extremely strong poison which, in small doses, is capable of killing healthy animals.

Since the time when the botulinus toxin was discovered by van Ermengem, numerous experiments have been made on the extraction of the pure toxin and the study of its nature. These experiments, however, have not met with success, since the majority of those working with this toxin have been unable to purify it of substances associated with it.

Briner and Kemper attempted to extract the toxin by precipitation of proteins with sodium sulfate, but these experiments gave a negative result because of large losses of the toxin. E. and F. Sommer produced dry preparations of the toxin by means of adsorption on alumina hydrate and on kaolin, but these preparations contained enzymes of the triptase type.

Later Schübel succeeded in extracting the toxin by way of dialysis, but the dialysate showed the biuret reaction. Schübel's lack of success was due to his use of an ultrafiltrate which apparently contained soluble proteins capable of passing through an ultrafilter.

Dozier, Wagner and Meyer suppose that the toxin is liberated as a result of autolysis of the bacterial cell. On the basis of their experimental findings, they arrived at the conclusion that in the period of initial multiplication on an alimentary medium (the first 15 to 20 hours of growth), no toxin is liberated, because there are no cells dying off.

In Meyer's opinion, the carrier of the toxin is a protein (nucleoprotein) with an isoelectric point at pH 4.4. All the dry toxins produced in this author's laboratory showed the biuret reaction for protein. Weinberg, and also Meyer's associates Levinson and Bronfenbrenner, consider it possible that the mole-

cule of the toxin consists of two groups, namely, a toxophoric group, the carrier of toxic principle, and a haptophoric group, which binds the antitoxin. The former is not very stable; the latter is comparatively stable and may persist unaltered for a long time.

In the research here described, we attempted to obtain botulinus toxin in dry form and to study its properties. In addition, our intention was to establish the relationship between products of the decomposition of carbohydrate substances and the process of toxin-formation in B. botulinus; likewise to discover whether this toxin was an endotoxin or an exotoxin.

For growing bacillus botulinus cultures and for the production of the toxin, we employed green peas with 20% glucose.

We preferred to work with a vegetable medium because with this type of culture medium we always obtained a stronger toxin than with the usual meat-peptone broth. Working with a vegetable medium is also more convenient, since vegetable proteins have been better studied. For our research, we took B. botulinus Type A (American strain 180).

The method of extracting the toxin was as follows. 100 ml of the culture were vacuum-distilled at a temperature below 40°C so that the toxin would not be destroyed in the process of separating the volatile substances. The residue, after distillation, was brought back to its original volume with physiological solution and tested for toxicity.

Here we found that if the titer of the toxin before distillation was 0.00001 ml, then after distillation it would be considerably increased and would go up to 0.0000001 ml. This increase in the titer may be explained as due to cells of B. botulinus being destroyed during the distillation, with the result that toxin is liberated into the surrounding medium. This was confirmed in the next experiment, in which the culture, before distillation, was filtered through a double-layered collodion bag to remove bacterial cells. Upon vacuum-distillation of this ultrafiltrate, at a temperature less than 40°C, no increase in the toxin titer was observed.

This is grounds for concluding that the toxin of the B. bacillus is an endotoxin, which is released to the surrounding medium when bacterial cells are destroyed.

In our tests, we always found the toxin to be already present after a 15-hour incubation; this disagrees with the findings of Dozier, Wagner and Meyer, quoted above. The table shows the lethal dose of the toxin, for mice, in a culture on

meat-peptone broth, at the end of 15 hours, and then at the end of each successive 24 hours.

Duration of growth (hrs)	Lethal dose of toxin (ml)	No. of mice
15	0.0001	2
40	0.00001	2
64	0.000001	2
88	0.000001	2
112	0.000001	2
136	0.000001	2
160	0.0000001	2
182	0.0000001	2
208	0.0000001	2
232	0.0000001	2
258	0.000001	2

These data are evidence that toxin is already present in the culture after 15 hours of bacterial growth, and in quite large amounts. Of course, the toxin titer continues to rise as the culture grows and ages; only on the tenth or eleventh day does it begin to fall. It is therefore impossible to agree with the opinion of the above-mentioned authors, namely that in a young culture there are no bacterial cells dying.

According to the findings of Topley and Wilson the development of bacteria in an alimentary medium takes place in four phases. The first phase lasts 2-3 hours, during which time the multiplication proceeds very slowly. In the second phase, the multiplication of the bacteria proceeds in a geometric progression, and this period lasts 5-6 hours. Then, in the third phase, there begins a period of throttling-down, in which the bacteria gradually lose their initial speed of multiplication. This latter period lasts from the 8th or 9th hour to the 12th or 13th hour from the start of the incubation. Finally there occurs the fourth phase, in which the bacteria gradually decrease in numbers and enter upon a period of more mortality.

According to this, the period in which the bacteria begin to die off en masse will begin 12 or 14 hours from the start of incubation, which is completely in accord with our findings on the formation of toxin in a culture of B. botulinus after 15 hours of incubation.

For the extraction of the pure toxin, we employed two methods.

The first method is based on the extraction of the albumin and globulin fractions of the proteins by salting-out with ammonium sulfate and sodium chloride. The washed protein is then placed in the dialyser (a double-layered collodion bag) and dialysed. The water is changed every two days and the amounts of toxin, ammonium sulfate and sodium chloride in this water are determined. The amount of ammonium chloride is found by titration with $Ba(OH)_2$ solution, and the amount of sodium chloride by titration with silver nitrate.

The second portion of the dialysate did not contain any ammonium sulfate or sodium chloride, but there was toxin in it. The ammonium sulfate and sodium chloride, as crystalloids, had gone with the first portion of the water. The toxin on the other hand dialysed slowly, and thus could be detected after the ammonium sulfate and sodium chloride had been removed.

The toxin thus extracted did not show the biuret reaction for protein; it was neutralised by antibotulinus serum. In addition to the biuret reaction, we also carried out the xanthoprotein reaction and Milon and Pauli's reaction to test for protein.

However, all these tests were negative. The dialysates obtained were toxic, killing mice at a concentration of 0.0001 ml.

50 ml of the dialysate were evaporated in a vacuum-dryer at room temperature. 5 mg of the dried toxin thus obtained were dissolved in 5 ml of physiological solution and tested for toxicity. The toxin in the dry form had not suffered any alteration.

The original material had a titer of 0.0001 ml. After evaporation and re-dissolving in physiological solution, the toxin titer was 0.0001 ml, that is, ten times greater, which corresponds to the decreased volume.

We attempted to precipitate proteins along with the toxin by using alcohol and acetone and then subjecting the residue to dialysis, since alcohol and acetone can easily be removed,

but it was found that both the alcohol and acetone partially destroyed the toxin and, in consequence, the dialysate obtained had a very low titer (0.5 ml).

In the second method, 500 ml of the culture were subjected to ultrafiltration in a double collodion bag to rid it of protein. The toxin in the ultrafiltrate was precipitated with trichloracetic acid; after centrifuging and washing with sulfuric ether, we would obtain, from the trichloracetic acid, 50 mg of the toxin. The ultrafiltrate showed the biuret reaction for protein, but the toxin precipitated from this ultrafiltrate, after being dissolved in water, would not give this reaction. It seems that soluble proteins go through the double collodion bag and are not precipitated by trichloracetic acid.

Testing of the toxin here obtained showed that it too would preserve its original titer.

Toxin obtained by both these methods had the same titer, which demonstrates that the two preparations are identical.

From all the above, we may conclude that B. botulinus toxin is a specific isolable substance not bound to a protein.

In the culture, the toxin is mechanically bound to a protein molecule, and after dialysis through a collodion bag, it passes into the dialysate. All findings controvert the old notion of its protein nature. The toxin produced by us does not dissolve in acetone, ether, or alcohol, but is partially destroyed by acetone and alcohol; it dissolves with difficulty in water, and loses its toxicity when acted upon by dilute alkali.

CONCLUSIONS

1) Our findings establish that the toxin of the B. botulinus is not of a protein nature. The toxin is mechanically bound to protein substances, upon dialysis of which it passes into the dialysate.

2) The toxin of B. botulinus is an endotoxin, and is released upon destruction of the bacterial cell.

3) Botulinus toxin is insoluble in acetone, ether and alcohol; it is soluble with difficulty in water, and is neutralized by a weak alkali.

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